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Effect of age, breed and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs

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Abstract

The focus of this study was to examine the influence of age and diet on various parameters of immune function in young and old Fox Terriers and Labrador Retrievers. Eighteen young and old dogs were utilized for this study. Young and old dogs were fed a basal diet containing an (n-6) : (n-3) ratio of 25 : 1 for sixty days (Phase I). Half of the dogs were then switched to a diet with an (n-6) : (n-3) ratio of 5 : 1, and all were maintained on their respective diets for an additional sixty days (Phase II). Results from these studies revealed an age-associated decline in several immune parameters measured. Both these breeds demonstrated a reduction in sheep red blood cell titers, as well as in their ability to respond to different mitogens. Interestingly, this decline was greater in Fox Terriers, suggesting a decrease in cellular proliferative capacity in lymphocytes isolated from the larger breed. Neither cytokine production or DTH response was affected by age. Diet and breed interactions resulted in a significant increase in T- and B-cell mitogen responsiveness. In contrast, supplementation with n-3 fatty acids did not affect IL-1, IL-6 or TNF-α production. Supplementation with n-3 fatty acids resulted in increased PGE_3 production from peritoneal macrophages but had no effect on PGE_2 production from peripheral blood mononuclear cells or peritoneal macrophages. The n-3 fatty acid supplementation did not influence α-tocopherol status although older dogs had significantly lower serum α-tocopherol concentrations. Oxidative status

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of these dogs was assessed by serum levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Feeding an n-3-enriched diet did not affect 4-HNE levels but significantly decreased MDA levels in old dogs. In summary, this study indicates that feeding a diet containing an (n-6) : (n-3) fatty acid ratio of 5 : 1 had a positive, rather than a negative, effect on the immune response of young or geriatric dogs

Keywords: Canine; Omega-3; Omega-6; Prostaglandin; Age; Immune response; Vitamin E; Lipid peroxidation; Eicosanoids; Cytokine

1. Introduction

The interaction between dietary fat and the immune response has been an area of growing interest. Omega-3 (n-3) fatty acids have been used in veterinary medicine as supplements for pruritic patients to control the inflammatory response that is associated with the disease (Miller et al., 1993; Paradis et al., 1991; Scott and Bueger, 1988). The influence of n-3 fatty acids on inflammation has been suggested to be through the incorporation of these fatty acids into cellular membranes and serving as substrates for eicosanoid metabolism, resulting in the production of eicosanoids with lower inflammatory potential than those produced from omega-6 (n-6) fatty acids (Reinhart, 1996). Recent studies have demonstrated that eicosanoid metabolism can be altered in the dog with dietary adjustments of n-6 to n-3 fatty acids (Vaughn et al., 1994). These studies have led to widespread incorporation of n-3 fatty acids in commercial pet foods.

Increased utilization of n-3 fatty acids has heightened the interest in the interaction between these fatty acids and the immune response. Studies examining this interaction have reported mixed results. While some studies have demonstrated an increase in T-cell mitogen response due to n-3 feeding (Levy et al., 1982; Payan et al., 1986), others have reported a decline in T-cell mediated immunity as well as in the production of interleukin (IL)-1, IL-2, IL-6 and tumor necrosis factor (TNF)B-α (Meydani M. et al., 1991a; Santoli and Zurier, 1989). In dogs, it has been demonstrated that feeding diets containing an (n-6) : (n-3) ratio of 1.4 : 1 results in a decline in delayed type hypersensitivity responsiveness (DTH) which is an in vivo measure of T-cell immunity (Wander et al., 1997). Feeding diets containing n-3 fatty acids has been reported to result in decreased serum vitamin E concentrations and increased serum lipid peroxidation markers (Meydani et al., 1991; Wander et al., 1997). These changes have been suggested to contribute to the decline in T-cell responsiveness in the presence of n-3 fatty acids. Studies have suggested that the negative aspects of n-3 fatty acid supplementation may be negated by vitamin E supplementation (Kramer et al., 1991).

A negative interaction between n-3 fatty acids and the immune response would be of particular concern for the geriatric population. The negative influence on the immune response may be more pronounced in the elderly (Levy et al., 1982). The aging process has been associated with a decline in immune responsiveness in a variety of species. The age-associated changes in the immune response have mainly been attributed to a decrease in responsiveness to T-cells (Miller, 1989; Makinidan, 1981). A decline in immunological vigor has been demonstrated in the dog. Laboratory studies have observed a decrease in

mitogen stimulation (Sheffy et al., 1985; Greeley et al., 1996), chemotaxis and phagocytosis (Tizard and Warner, 1987). A recent longitudinal study has reported age-related changes in clinical immunological parameters, such as decreased numbers of white blood cells and immature neutrophils and increased numbers of mature neutrophils and concentration of immunoglobulin G (Strasser et al., 1993). Together, these data demonstrate an age-related decline in immunity in dogs that may increase the susceptibility of the geriatric animal to infection (Mosier, 1989).

Few studies have been conducted to examine the influence of various (n-6) : (n-3) ratios on the immune response of young and old dogs (Wander et al., 1997). The purpose of this study was to examine the influence of diets formulated to contain (n-6) : (n-3) ratios of 25 : 1 or 5 : 1 on the immune response and oxidative status of young and old Fox Terriers and Labrador Retrievers. A secondary objective of this study was to determine if the effect of age on the immune response differs between different breeds.

2. Materials and methods

2.1. Diet

The formulation of the diet is presented in Table 1. The (n-6) : (n-3) fatty acid ratio of the diets was 25 : 1 for the control diet and 5 : 1 for the test diet. The choice of the 5 : 1 (n-6) : (n-3) ratio was influenced by the fact that this ratio was demonstrated to decrease LTB_4 synthesis while increasing LTB_5 synthesis by neutrophils and skin of the dogs (Vaughn et al., 1994).

Table 1
Diet composition

Ingredient	(n-6) : (n-3) fatty acid ratio	
	25 : 1%	5 : 1%
Corn	27.00	27.00
Rice	26.00	26.00
Chicken protein	26.15	26.15
Chicken fat	8.21	6.50
Beet pulp	4.00	4.00
Egg	2.50	2.50
Chicken digest	2.00	2.00
Fish Oil	—	1.65
Yeast	1.00	1.00
Mineral mix[a]	2.91	2.71
Vitamin mix[b]	0.27	0.27
Ground flax seed	—	0.24

[a] Diet was formulated to contain a final concentration of 11 000 ppm Ca, 8000 ppm P, 2400 ppm choline, 5500 ppm Na, 8000 ppm Mg, 200 ppm Zn, 400 ppm Fe, 25 ppm Cu, 3.5 ppm I, 800 ppm Mn, 0.2 ppm S, and 0.4 ppm Se.

[b] Diet was formulated to contain a final concentration of 15 kIU/kg Vitamin A, 100 IU/kg, Vitamin E, 1200 IU/kg Vitamin D, 13 mg/kg thiamine, 50 mg/kg niacin, 25 mg/kg pantothenate, 5 mg/kg pyridoxine, 0.3 mg/kg biotin, and 1.0 mg/kg folic acid.

2.2. Animals

Eighteen young (9 Labrador Retrievers, mean age 1.5 years; 9 Fox Terriers, mean age 1.8 years) and eighteen adult (9 Labrador Retrievers, mean age 9.6 years; 9 Fox Terriers, mean age 11.5 years) dogs were utilized for this study. During Phase I, all dogs were fed a basal diet containing an (n-6) : (n-3) ratio of 25 : 1 for eight weeks. During Phase II, half of the dogs were switched to a diet containing an (n-6) : (n-3) ratio of 5 : 1 and all dogs were fed their respective diets for the following eight weeks. Blood was drawn from each animal at the end of each respective feeding period.

2.3. Isolation of peripheral blood mononuclear cells

Venous blood was collected into heparinized tubes and diluted threefold with RPMI 1640 medium (GibcoBRL, Grand Island, NY). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Hypaque gradients (Pharmacia Biotech, Piscataway, NJ). The fraction containing mononuclear cells was isolated, washed twice, and re-suspended in the RPMI 1640 medium. These cells were then used either to measure mitogen-induced lymphoproliferative responsiveness or cytokine (IL-1, IL-6, TNF-α) levels. For cytokine induction, cells were cultured in RPMI-1640 medium in the presence of 10 μg/ml of LPS (Sigma, St. Louis, MO) for 24 h. The culture supernatant fluids were collected and any non-adherent cells pelleted by centrifugation. The cell-free supernatants were frozen at −80°C until analysis and the non-adherent and adherent cells digested with 1N ammonium hydroxide and 0.2% Triton X-100 (Sigma, St. Louis, MO) for total cellular DNA quantification.

2.4. Isolation of peritoneal macrophages

Peritoneal cells were harvested by peritoneal lavage using buffered Hanks balanced salt solution. Peritoneal macrophages were re-suspended in RPMI 1640 medium containing 5 mg/ml bovine serum albumin (BSA)(Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine (GibcoBRL, Grand Island, NY) and cultured in 25 ml tissue culture flasks (Corning, Corning, NY) for 2 h at 37°C and 5% CO_2. The non-adherent cells were washed off with medium, and the remaining adherent macrophages cultured for 24 h in a medium containing 10 μg/ml of LPS. Culture supernatant fluids were collected and stored and the cells digested for total DNA as described above.

2.5. TNF bioassay

Assay for TNF-like activity was performed on L929 murine fibroblasts. L929 cells were grown in an RPMI 1640 medium supplemented with 1×10^5 U/l penicillin, 6.9×10^{-5} mol/l streptomycin, 2×10^{-3} mol/l L-glutamine and 10% fetal calf serum (FCS) (Hyclone, Logan, UT). Dilutions of peritoneal macrophage or PBMC culture supernatant fluid (1 : 50 or 1 : 100) were added in octuplicate to 96-well culture plates containing L929 murine fibroblasts. Medium supplemented with actinomycin-D (3.98×10^6 mol/l) was added to each of the wells. After incubation for 20 h at 37°C,

the fluid in the wells was discarded, and 75 µl of 7.24×10^{-7} mol/l (3-[4,5 dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) (MTT) (Sigma, St. Louis, MO) in Hanks balanced salt solution added to each well. After 3 h, 100 µl of a solution of 6.9×10^{-1} mol/l SDS in 6.45 mol/l *N,N* dimethylformamide (pH 4.7) (Sigma, St. Louis, MO) were added to each well and incubated at 37°C overnight (Hansen et al., 1989). The absorbency at 550 nm was then determined using a microplate reader. To determine the amount of TNF-like activity, a standard curve was produced for each assay using human recombinant TNF-α (Genzyme, Boston, MA).

2.6. IL-6 Bioassay

The IL-6 bioassay was performed using B9-cells (Aarden et al., 1987). The cells were grown in Iscove's Modified Dulbecco's medium (GibcoBRL) supplemented with 10% (FCS), 40 pg/l of mouse recombinant IL-6, and 50 mM 2-mercaptoethanol (2-ME) (Sigma, St. Louis, MO). Dilutions of PBMC or peritoneal-macrophage culture supernatant fluid (1 : 100 or 1 : 200) were added in octuplicate to 96-well culture plates containing B9-cells. After 72 h of incubation, 20 µl of 7.24×10^{-7} mol/l MTT in HBSS were added to each well and then, 6 h later, 100 µl of SDS-DMF. The absorbency at 550 nm was recorded after an overnight incubation at 37°C. A standard curve using recombinant mouse IL-6 (Genzyme, Boston, MA) was used to determine the amount of IL-6 bioactivity.

2.7. IL-1 bioassay

A mouse plasmacytoma cell line (T1165.17) that proliferates in response to IL-1 was used to assay for IL-1 activity. The IL-1 receptor on the T1165.17 cell line was blocked using a monoclonal antibody (LA 15.6) to the IL-1 receptor. The cell line and antibody were generously supplied by Dr. Andrew Glasebrook (Eli Lilly, Indianapolis, IN). The cells were grown in minimal essential medium supplemented with 10% FCS, 50 mM 2-ME, and 200 pg/l of IL-1β. Cells were seeded in 96-well plates (5×10^5/ml), and samples assayed in quadruplicate. Four wells were blocked with antibody (1 µg/ml) 30 min before addition of samples and four wells were unblocked. Samples diluted from 1 : 100 to 1 : 200 were added and incubated at 37°C for 18 h, and then MTT added as described for the IL-6 bioassay. Six hours later, SDS-DMF was added and the plates incubated and absorbency measured as described for the IL-6 bioassay. The difference in absorbency between blocked and unblocked wells, corrected for control levels, determined the amount of IL-1 activity in the culture medium. A standard curve was generated for each assay using mouse recombinant IL-1β Genzyme, Boston, MA).

2.8. Mitogen-induced lymphocyte proliferation

Peripheral blood mononuclear cells (1×10^5) were dispensed in quadruplicate into individual wells of a 96-well microtiter plate (Costar, Corning, NY) for culture with three concentrations of either Concanavalin A (Con A), Phytohemagglutinin-P (PHA), or Pokeweed mitogen (PWM)(GibcoBRL). Cells were cultured at 37°C, 5% CO_2 for 72 h. Eighteen hours prior to termination, cells were pulsed with 1 µCi of tritiated thymidine (ICN

Radiochemical, Irvine, CA). After being harvested onto glass fiber filters, incorporation of tritiated thymidine was determined by liquid scintillation spectroscopy using a Beckman scintillation counter. Results are presented as corrected CPMs (cCPM), which is the average counts per mitogen-stimulated cultures—average counts of unstimulated cultures.

2.9. *In vivo antibody production*

On Day 60 following maintenance on the Phase II diet ((n-6) : (n-3) ratio of 5 : 1), dogs were intramuscularly injected with 5 ml of 10% suspension of sheep red blood cells (S-RBC)(Sigma). Blood samples were taken prior to injection as well as on days 7, 14 and 21 post-injection. Serum was separated by centrifugation and frozen at −20°C until analysis. Serum antibody titers to sheep red blood cells were determined according to the procedure of Wengman and Smithies (1966). Data was expressed as the log base 3 of the reciprocal of the titer dilution.

2.10. *Oxidative stress markers*

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations in the serum were determined by using a commercial spectrophotometric kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

2.11. *Cholesterol and triglyceride analyses*

Serum samples were analyzed by a Cobas Mira Analyzer (Roche Diagnostics Systems, Somerville, NJ).

2.12. *Vitamin E analysis*

Serum vitamin E was analyzed by the HPLC method described previously (Meydani et al., 1989). Tocol (gift from Hoffmann–LaRoche, Nutley, NJ) was used as an internal standard (Handelman et al., 1988), and eluted peaks were detected with a Perkin–Elmer 650-15 fluorescence spectrophotometer (Norwalk, CT).

2.13. *Prostaglandin analysis*

Concentration of PGE_2 and PGE_3 in supernatants were analyzed using a modified method of Reilly and Blair (Reilly et al., 1988; Blair et al., 1993). Unless otherwise stated, chemicals mentioned were obtained from Sigma. Briefly, 250–500 μl of supernatant were spiked with an internal standard (PGE2-D4; Cayman Chem). After 15 min, 200 μl of methoxyamine (0.5 g/ml) and 30 μl of 88% formic acid (Fisher Scientific, Pittsburgh) were added to the sample and allowed to stand for an additional 15 min. During this time a solid-phase extraction cartridge (Isolute C-18, Jones Chromatography, Lakewood, CO) was prepared by passing 2 ml of ethanol and 2 ml of distilled water through the cartridge. The sample was placed on the cartridge and washed with 2 ml of distilled water followed by 1 ml of hexane. The prostaglandin fraction was collected with 1 ml of methanol and the collected fraction

was dried completely under nitrogen. At the completion of drying, the sample is reconstituted with 100 μl acetonitrile, 20 μl *n,n*,diisopropylethylamine and 20 μl pentafluorylbenzyl-bromide, vortexed, and allowed to react for 15 min. The sample is dried under nitrogen, reconstituted with 30 μl methanol and placed on a thin layer chromatography plate (Whatman LK6D silica gel, 5 × 20 cm 60A, 25 μm; Whatman, Clifton, NJ) preconditioned by heating at 120°C for 1 h. Plates were developed in a tank with 50 ml 20% heptane/ethyl acetate. Regions of interest were scraped from each channel, placed in a centrifuge tube after identification by comparing migration patterns to a thin-layer chromatography plate run with 2 mg of derivitized compounds of interest and visualized by charring with 5% $CuSO_4$. Thirty microliters of distilled water and 1 ml of ethyl acetate were added to each sample tube, vortexed, and ethyl acetate was decanted to a second tube. Another 0.5 ml of ethyl acetate was added to the sample, vortexed, and decanted. The combined ethyl acetate was dried under N_2 and the sample was reconstituted with 20 μl pyridine and 20 μl *bis*-(trimethylsily) triflouroactemide. The sample was then vortexed and allowed to react for 15 min, then dried under N_2. The sample was reconstituted with 20 μl of dodecane, vortexed, and transferred to an autosampler vial. Samples (1–4 μl) were injected and separated on a HP 5890 Series II GC HP 5989B ms engine (Little Falls, DE) with a HP5MS column (30 m, 0.25 mm, 0.25 μm) with an H_2 carrier gas flow rate of 3 ml/min. The mass spectrometer conditions were set at a source temperature of 150°C and quad at 100°C. PGE_2, PGE_3 and PGE_2-D4 peaks were identified under conditions established with known standards (PGE_2 524 mass : charge ratio; *m/z*), 8.7 min run time; PGE_3 522 *m/z*, 8.5 min run time; and PGE_2-D4 528 *m/z*, 8.6 min run time).

2.14. Delayed-type hypersensitivity test (DTH)

Hindquarters were shaved from each animal prior to injection with an area ≈2.5″ × 5″. Injection sites were marked with a felt pen and a paper card with the preset holes. Injection sites were swabbed with alcohol. One hundred microliters of Con A (0.05%), or saline were injected intramuscularly. Data was recorded as skinfold thickness at time intervals of 24, 48 and 72 h post-injection.

2.15. Statistical analysis

Statistical analysis was conducted using the SAS statistical program (Release 6.03, 1988, SAS Institute, Cary, NC). Data was analyzed by a 2 × 2 factorial two-tailed analysis of variance with individual differences analyzed by single degree-of-freedom comparison using Fischer's least significant difference procedure and are reported as mean ± standard error. Significance was set at $p < 0.05$.

3. Results

3.1. Age and breed interactions

Data presented in Fig. 1 illustrates that PBMCs obtained from Fox Terriers and Labrador Retrievers exhibit an age-associated decline in their ability to proliferate in

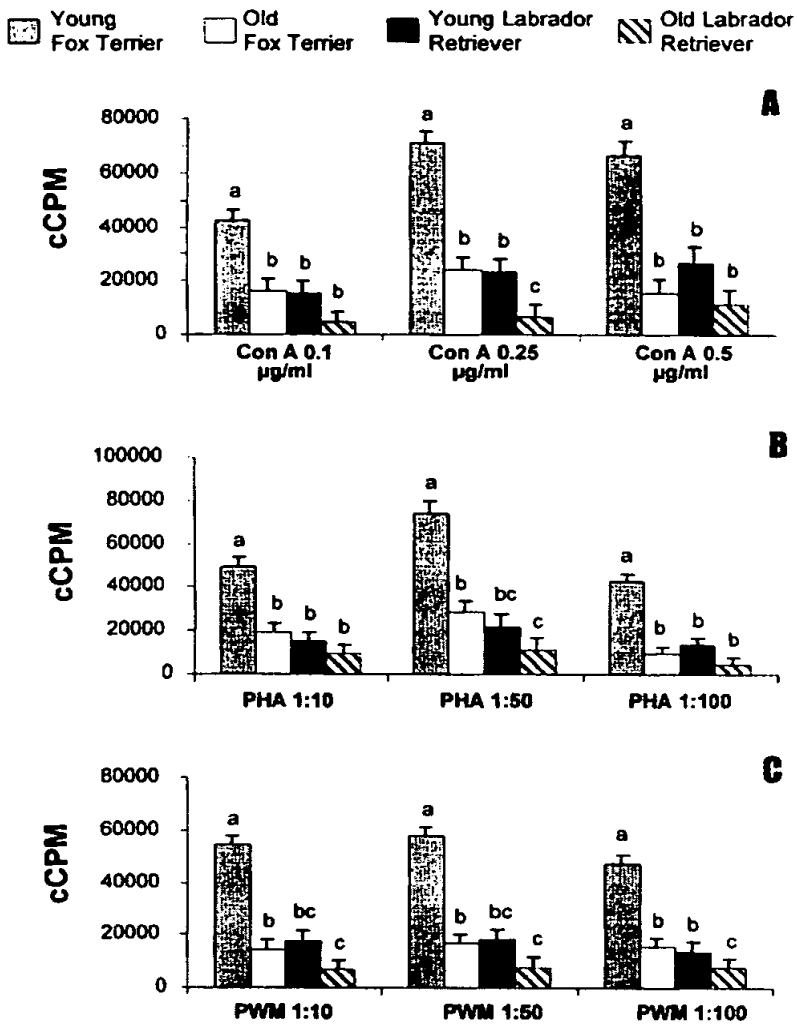

Fig. 1. Effect of breed and age on mitogen response of peripheral blood mononuclear cells from dogs. Peripheral blood mononuclear cells were isolated from heparinized venous blood via density gradient centrifugation over Ficoll–Hypaque gradients. 1×10^5 cells were cultured in quadruplicate in 96-well plates in the presence of media: (A) Con A (0.1; 0.25 and 0.5 µg/ml), (B) PHA (1 : 10, 1 : 50 or 1 : 100 dilution); or (C) PWM (1 : 10, 1 : 50 or 1 : 100 dilution). Results are presented as corrected CPMs (cCPM), which is the average counts per mitogen-stimulated cultures—average counts of unstimulated cultures. Data are expressed as the means ± SE. Means within the same mitogen concentration that do not share the same superscript are significantly different ($p < 0.05$).

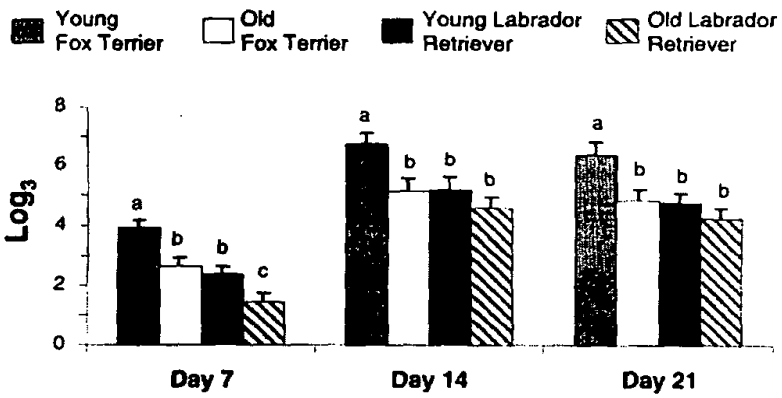

Fig. 2. Effect of breed and age on antibody production to sheep red blood cells. All dogs were administered a 5-ml intramuscular injection of a 10% solution of sheep red blood cells. Blood was collected from these dogs on days 0 (background) 7, 14 and 21 post-injection. Serum titers were determined via a hemagglutination assay. Data is expressed as mean ± SE of the log base 3 of the inverse of the titer. Means within the same day that do not share the same superscript are significantly different ($p < 0.05$).

response to Con A (Fig. 1(A)), PHA-P (Fig. 1(B)), and PWM (Fig. 1(C)). It is interesting to note that the degree of decline was greater for Fox Terriers than Labrador Retrievers. Also, thymidine incorporation was greater in lymphocytes isolated from Fox Terriers than Labrador Retrievers.

The humoral immune response, measured as antibody titer to S-RBCs, was also affected by age. There was a significant reduction in antibody titer in old vs. young Fox Terrier dogs at 7, 14 and 21 days post-immunization (Fig. 2). In contrast, although old Labrador Retrievers demonstrated lower antibody concentration at each time interval, significance was observed only on Day 7 (Fig. 2). The DTH skin response to PHA, used as an indicator of changes in cellular immunity, revealed no significant age differences (data not shown).

Pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, mediate the response of the host to inflammation. Stimulation of either peripheral blood mononuclear cells or peritoneal macrophages obtained from young and old Fox Terriers and Labrador Retrievers with lipopolysaccharide (10 μg/ml) resulted in no significant differences in TNF-α, IL-1 or IL-6 bioactivity (data not shown).

3.2. Diet and breed interactions

Data presented in Fig. 3 illustrates that switching the diet to an (n-6) : (n-3) fatty acid ratio of 5 : 1 resulted in a significant increase in mitogenic response to Con A, PHA and PWM in young Fox Terriers and Labrador Retrievers. This was a consistent finding independent of mitogen concentration. Older animals, however, exhibited no significant change to any of the mitogens evaluated subsequent to switching these animals to the 5 : 1 diet. Interestingly, older dogs exhibited significantly lower mitogenic responses than

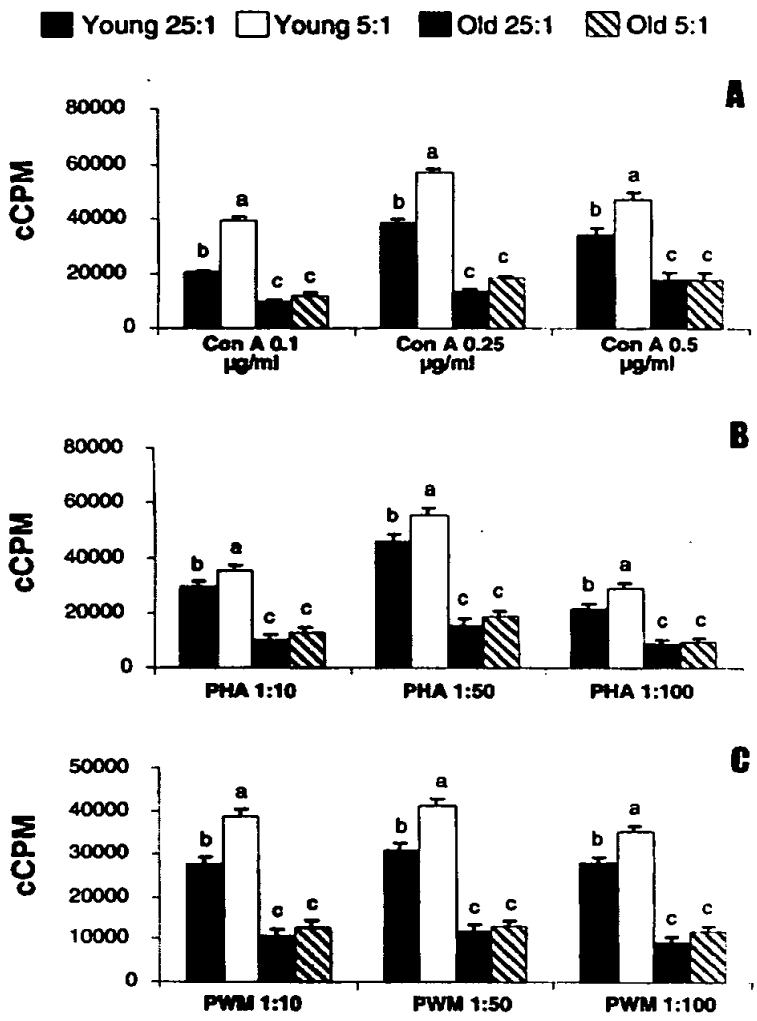

Fig. 3. Effect of n-6 : n-3 fatty acid ratio in the diet on mitogen response of peripheral blood mononuclear cells from dogs. Peripheral blood mononuclear cells were isolated from heparinized venous blood via density gradient centrifugation over Ficoll–Hypaque gradients. 1×10^5 cells were cultured in quadruplicate in 96-well plates in the presence of media: (A) Con A (0.1; 0.25 and 0.5 µg/ml); (B) PHA (1 : 10, 1 : 50 or 1 : 100 dilution); or (C) PWM (1 : 10, 1 : 50 or 1 : 100 dilution). Cells were cultured at 37°C, in 5% CO_2 for 54 h. Cells were then pulsed with 1 µCi of tritiated thymidine and incubated for an additional 18 h. PBMCs were then harvested on fiberglass filters and thymidine incorporation into the DNA was determined via scintillation spectroscopy. Results are presented as corrected CPMs (cCPM), which is the average counts per mitogen-stimulated cultures—average counts of unstimulated cultures. Data are expressed as the means ± SE. Means within the same mitogen concentration that do not share the same superscript are significantly different ($p < 0.05$).

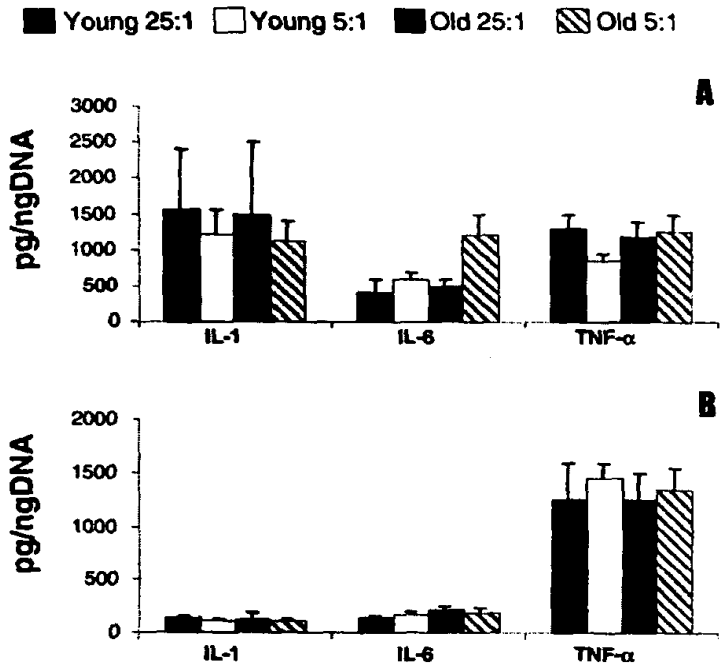

Fig. 4. Effect of n-6 : n-3 ratio in the diet on IL-1, IL-6 and TNF-α production from peritoneal cells (A) or PBMs (B) from dogs. Two million adherent peritoneal cells or 5×10^6 PBMCs were cultured at 37°C, 5% CO_2 in RPMI 1640 containing 10 μg/ml LPS. Cells were cultured for 24 h at which time supernatant was collected and stored at −80°C until analysis. Cells were digested with 1 N ammonia hydroxide and 0.2% Triton x-100 for DNA quantitation. IL-1, IL-6 and TNF-α activity in the supernatant was measured by bioassay. Data is expressed as mean ± SE.

young dogs to both, T- and B-cell mitogens, a fact that was not affected by switching from a low to a high n-3 diet.

The effect of n-3 fatty acid supplementation on the production of pro-inflammatory cytokines is shown in Fig. 4. Feeding a diet containing an (n-6) : (n-3) ratio of 5 : 1 had no significant effect on IL-1, IL-6 or TNF-α production by peritoneal macrophages (Fig. 4(A)) or peripheral blood mononuclear cells (Fig. 4(B)). In addition, omega-3 supplementation also did not influence sheep red blood cell antibody titer response (Fig. 5).

In order to determine if feeding a 5 : 1 ratio affects eicosanoid production, peritoneal macrophages and peripheral blood mononuclear cells were stimulated with LPS (10 μg) to stimulate prostaglandin production. Neither age nor diet affected PGE_2 production from either macrophages or PBMC (Table 2). Interestingly, there was a significant difference observed in PGE_3 production in PBMCs of young and aged dogs fed the high n-3 diet (Table 2).

There was a significant increase in serum cholesterol, triglycerides, and total lipids observed in aged dogs fed a diet containing an (n-6) : (n-3) fatty acid ratio of 5 : 1

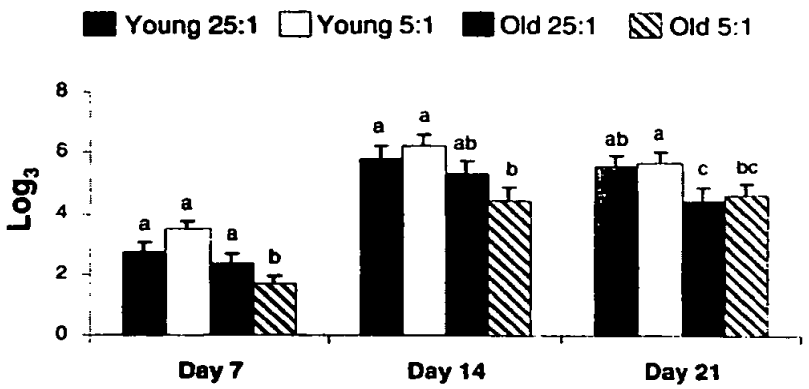

Fig. 5. Effect of n-6 : n-3 fatty acid ratio in the diet on antibody production to sheep red blood cells. All dogs were administered a 5-ml intramuscular injection of a 10% solution of sheep red blood cells. Blood was collected from these dogs on days 0 (background) 7, 14 and 21 post-injection. Serum titers were determined via a hemagglutination assay. Data is expressed as mean ± SE of the log base 3 of the inverse of the titer. Means within the same day that do not share the same superscript are significantly different ($p < 0.05$).

Table 2
Effect of omega-6: omega-3 fatty acid ratio on cellular eicaosanoid production, serum cholesterol, triglycerides, α- and γ-tocopherol, MDA and 4-HNE levels in the young and old dogs[a,b]

Measurement	Young 25 : 1	Young 5 : 1	Old 25 : 1	Old 5 : 1
PBMC PGE_2 (ng/mg DNA)[c]	160.09 ± 16.24a	159.85 ± 14.23a	85.55 ± 16.24b	118.31 ± 14.33b
PBMC PGE_3 (pg/mg DNA)	94.53 ± 138.28a	547.29 ± 146.67b	59.04 ± 156.80a	782.75 ± 138.28b
Mϕ PGE_2 (ng/mg DNA)[d]	131.96 ± 12.87	132.29 ± 11.51	114.47 ± 12.87	108.61 ± 12.13
Mϕ PGE_3 (pg/mg DNA)	197.55 ± 78.77	205.35 ± 70.46	330.61 ± 78.77	241.04 ± 74.27
Cholesterol (mg/dl)	254.44 ± 4.74ab	242.11 ± 4.74a	266.12 ± 5.03b	294.00 ± 4.74c
Triglycerides (mg/dl)	38.11 ± 2.33a	40.17 ± 2.33a	57.51 ± 2.47b	76.72 ± 2.33c
Total lipid (mg/dl)[e]	292.56 ± 6.40a	282.28 ± 6.40a	323.63 ± 6.79b	370.72 ± 6.40c
α-Tocopherol (μmol/mg total lipid)	8.27 ± 0.39ab	9.07 ± 0.39a	7.38 ± 0.42b	7.40 ± 0.40b
γ-Tocopherol (μmol/mg total lipid)	0.27 ± 0.02a	0.29 ± 0.02a	0.32 ± 0.03a	0.21 ± 0.02b
MDA (μmol/mg total lipid)	0.45 ± 0.02a	0.45 ± 0.02a	0.37 ± 0.02b	0.29 ± 0.02c
HNE (μmol/mg total lipid)	0.35 ± 0.08	0.33 ± 0.07	0.37 ± 0.09	0.37 ± 0.09

[a] Values are means ± SE. $n = 9$.
[b] Means within the same row that do not share the same letters are significantly different ($p < 0.05$).
[c] 1×10^6 PBMCs were cultured in RPMI-1640 in the presence of 10 μg/ml LPS for 24 h at 37 C in 5% CO_2. Supernatants were collected and frozen at −70°C.
[d] 2×10^6 Peritoneal cells were adhered to plastic Petri dishes for 90 min. Adherent cells were cultures in RPMI-1640 in the presence of 10 μg/ml LPS for 24 h at 37°C in 5% CO_2. Supernatants were collected and frozen at −70°C.
[e] Total lipid was calculated as the sum of cholesterol plus triglycerides.

(Table 2). The concentration of cholesterol was 10% higher than dogs who were fed an (n-6) : (n-3) fatty acid ratio of 25 : 1; whereas triglyceride and total lipids were 33% and 15% higher, respectively, in aged dogs fed the high n-3 diet.

Feeding dietary n-3 fatty acids have been suggested to increase the oxidative stress of the animal, possibly by decreasing vitamin E levels in the blood and tissue (Tappel, 1972). In our study, oxidative stress was assessed by measuring MDA and HNE. Both of these compounds are by-products of arachidonic acid metabolism and serve as markers for oxidative status of animals (Yu, 1993). There was no difference in HNE levels in young or old animals fed a high n-3 diet, but there was a significant difference in MDA in old dogs maintained on the 5 : 1 diet (Table 2). No significant difference in α-tocopherol levels was seen in young or aged dogs fed the high n-3 diet (Table 2). However, there was a 34% decrease in α-tocopherol levels (0.32 ± 0.03 vs. 0.21 ± 0.02; $p < 0.05$) in aged dogs fed the high n-3 diet (Table 2). It should be noted that these parameters were corrected for total lipid concentrations (triglycerides + cholesterol) since total serum lipids were observed to be higher in older dogs.

4. Discussion

The mammalian immune system can be significantly impacted by both, the aging process and nutritional factors such as dietary lipids (Bradley and Xu, 1996; Chandra and Kumari, 1994; Hannigan, 1994; Miller, 1989; Wick and Grubeck-Loebenstein, 1997). Aging has been demonstrated to influence both, the humoral and cellular immune function, as well as cytokine production (Greeley et al., 1996; Hobbs et al., 1993; Makinidan, 1981). Consequently, the aging immune system may contribute to the increased incidence of infectious disease and cancer. Dietary lipids have been reported to modulate the immune system either through regulating cytokines or eicosanoids (Blok et al., 1996; Calder, 1996, 1997; Miles and Calder, 1998; Yaqoob and Calder, 1995). Interestingly, results of these studies are equivocal, depending on the form of fatty acid used (n-6 vs. n-3), concentration added to the diet, and the species studied. To date, most research addressing the impact of aging and/or dietary lipids on immune function has been done in humans and rodents (Meydani S.N. et al., 1991; Payan et al., 1986). Little information is available regarding the effects of either of these variables on the canine immune system (Wander et al., 1997). The purpose of the present study was twofold; first, to determine whether the effect of age on immune function differs between different breeds, and second, what impact does feeding a diet supplemented with different ratios of n-6 to n-3 fatty acids have on the immune function, eicosanoid production, and lipid peroxidation.

Our results indicate that the mitogenic response of PBMCs obtained from aged Fox Terriers and Labrador Retrievers was significantly suppressed following stimulation with Con A, PHA and PWM when compared with young dogs. These findings are consistent with those presented in previous studies using Beagle or Labrador Retriever dogs (Davila et al., 1992; Greeley et al., 1996). In Beagles, however, suppression of lymphoproliferative responses was observed only with PHA, and not Con A (Davila et al., 1992). A key difference noted in our study was that the magnitude of this decline was greater in Fox Terriers as compared to Labrador Retrievers. Moreover, increased thymidine incorporation was the greatest in lymphocytes isolated from the small breed dog. This observed age-associated decline in mitogen response could reflect either a change in the pattern of

cytokines produced by $CD4^+$ T-cells (Hobbs et al., 1993), or a shift in the balance of naive cells to memory cells in aged animals (Nijhuis and Nagelkerken, 1992).

Cell-mediated immunity in Fox Terriers or Labrador Retrievers, detected as the DTH response to Con A, was not affected by age. Likewise, stimulation of peritoneal macrophages or adherent PBMCs with lipopolysaccharide resulted in no significant change in the levels of IL-1, IL-6 or TNF-α in these same dogs. There was, however, an age-associated decline in humoral immunity when expressed as a change in antibody titer to sheep red blood cells in young and aged dogs. This is in contrast to data presented in a previous study, in which no difference in antibody titer to keyhole limpet hemocyanin (KLH) was observed between young and old Labrador Retrievers (Greeley et al., 1996). Although the mean titer in old (9.4 years) dogs was less than that in young dogs, it did not represent a significant difference. These contradictory observations may simply reflect differences in the experimental protocols used, i.e. antigen dose, route of antigen administration, or sensitivity of the assay.

The results from our study indicate that supplementing the diet with an (n-6) : (n-3) fatty acid ratio of 5 : 1 had a significant effect on several indexes of the immune function. Mitogen responses to Con A, PHA and PWM were enhanced significantly in young dogs maintained on the high n-3 diet for sixty days. Older dogs exhibit a significantly lower mitogen-induced proliferative response, and although there was a trend toward increased lymphoproliferative responses when these animals were switched to the high n-3 diet, these differences were not significant. Our data does not agree with the previous studies in which subjects maintained on a diet supplemented with n-3 exhibit suppressed blastogenesis of mitogen-stimulated PBMCs (Meydani S.N. et al., 1991). These differences may be attributed to reduced levels of α-tocopherol brought on by the long-term administration of n-3 fatty acids (Meydani et al., 1991). Increased intake of vitamin E has been reported to restore n-3-induced suppressed blastogenic responses (Kramer et al., 1991). It is important to note that in our study, the vitamin E content in the diet was high enough to maintain plasma levels of vitamin E.

The effect of diet and breed interactions on the humoral immune response was not as evident as the age × breed interactions. The antibody titer against SRBCs in young dogs, when switched to an (n-6) : (n-3) ratio of 5 : 1 diet, increased on Day 7, 14 and 21 post-immunization. There were, however, no significant differences at any time point when compared with animals maintained on the (n-6) : (n-3) ratio of 25 : 1. Aged dogs had reduced antibody titers compared to young dogs when maintained on different diets with varying ratios of n-6 : n-3 fatty acids. In contrast to young dogs, aged animals exhibit a decrease in antibody titer when maintained on the high n-3 diet, with significance being observed on Day 7 post-immunization. The absence of any significant change in antibody titer in young animals maintained on a high n-3 diet might reflect a temporal change in the activity of antigen presenting cells. In a previous study, rats fed a high n-3 diet expressed diminished antigen presentation activity in the dendritic cell population (Sanderson et al., 1997). Dendritic cells are a principal cell type not only involved in T-cell activation, both in vivo and in vitro, but they are also required for T-cell-dependent antibody responses.

Pro-inflammatory cytokines, such as IL-1, TNF-α and IL-6, play a critical role in mediating the host response to infection and inflammation (Dinarello, 1992). It has been

proposed that supplementation of the diet with n-3 fatty acids may have therapeutic value in reducing inflammation associated with chronic debilitating illnesses, such as rheumatoid arthritis, colitis and psoriasis (Blok et al., 1996). The proposed mechanism for the mediation of this suppressive effect by dietary fatty acids is attributed to decreased production of pro-inflammatory cytokines. In our study, no differences were detected in IL-1, TNF-α, or IL-6 activity produced by either peritoneal macrophages or PBMC. These results contradict previously published reports evaluating the potential for diets rich in n-3 fatty acids to alter cytokine activity. For example, studies in humans have shown that the use of n-3 fatty acids in the diet decrease the production of pro-inflammatory cytokines (Endres et al., 1989; Meydani et al., 1991). In contrast, mice maintained on a diet high in n-3 fatty acids expressed increased cytokine activity (Hardardottir et al., 1992b; Lokesh et al., 1990). It is possible that these paradoxical responses observed in rodents and humans can, in part, be explained by the utilization of different experimental protocols as well as different cell types (Blok et al., 1996; Calder, 1997). In the canine model, it is possible that the dietary ratio of 5 : 1 is insufficient to alter cytokine activity. Many of the animal studies addressing the effects of dietary n-3 fatty acids on cytokines have used lower ratios of (n-6) : (n-3) to achieve the alteration in cytokine production (Hardardottir and Kinsella, 1991; Lokesh et al., 1990; Turek et al., 1991).

An alternative explanation for dietary lipids not altering cytokine activity in the canine model could be found in levels of eicosanoids produced by PBMC or peritoneal macrophages. It is known that eicosanoids regulate cytokine production by macrophages, and that supplementing the diet with fish oil results in decreased production of arachidonic acid-derived eicosanoids (Lokesh et al., 1986; Hardardottir and Kinsella, 1991, 1992a). In this study, there was no difference in PGE_2 levels detected in PBMCs or peritoneal macrophages. These data are consistent with those presented in a previous study in which no significant differences were detected in the concentration of PGE_2 in stimulated mononuclear cells obtained from geriatric beagles maintained on a low (33 : 1) or medium (5.4 : 1) fatty acid diet (Wander et al., 1997). There was a significant difference, however, in dogs fed a high (1.4 : 1) diet, as evidenced by a 52% reduction in PGE_2. This is in contrast to data on leukotriene production from neutrophil and skin in beagles in which feeding a diet with an (n-6) : (n-3) ratio of 10 : 1 or 5 : 1 resulted in decreased leukotriene B_4 production and increased LTB_5 production (Vaughn et al., 1994). This difference may be attributed to the examination of different eicosanoids or differences in tissues and/or cells examined. It should be noted that the reduction in PGE_2 synthesis from PBMCs of beagles fed a 1.4 : 1 fatty acid diet was associated with a decrease in DTH response signifying a depression in cell-mediated immune response.

Although there was no detectable difference in PGE_2 levels in young and old dogs maintained on the high n-3 diet, there was a significant difference in the PGE_3 levels observed in PBMCs of young and old dogs fed the high n-3 diets. This increase in PGE_3 production was not observed in the peritoneal macrophages isolated from dogs fed the high n-3 diet. This may be explained by the fact that PBMCs represent a heterogenous population of T- and B-cells as well as macrophages. In this population, macrophages are the main source for prostaglandin production (Goldyne and Stobo, 1982; Goldyne, 1986). However, it has been demonstrated that arachidonic acid (AA) released from lymphocytes

can be used to synthesize cyclo-oxygenase products (Samuelson, 1995). Feeding a diet high in n-3 fatty acids increases membrane composition of eicosapentanoic acid (EPA) (Hwang et al., 1988) which promotes the generation of 3-series PG (Knapp, 1990). It is possible that the increase in PGE_3 synthesis observed in PBMCs is due to the release of EPA instead of AA and subsequent utilization by the macrophages.

The beneficial effects of dietary n-3 fatty acids in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, and colitis, as well as heart disease are well documented (Blok et al., 1996; Herold and Kinsella, 1986; Glomset, 1985). Additionally, the therapeutic benefits derived from these diets have been attributed to an impact on immune function. Ironically, a potential harmful effect of these diets rich in n-3 fatty acids may be increased levels of lipid peroxidation, the products of which may contribute to the onset and development of age-associated diseases. In this study, the level of lipid peroxidation in young dogs was unaffected by diet. In contrast, aged animals demonstrated a decrease in MDA levels after being switched to the high n-3 diet. These results are not consistent with previously reported data. In a study conducted with young and older women, it was shown that supplementation of the diet with n-3 fatty acids resulted in an increase in MDA levels, and this increase was greater in older women (Meydani et al., 1991). More recently, it was reported that aged beagle dogs, maintained on a n-3 fatty acid diet of 1.4 : 1, exhibited a significant increase in plasma and urine TBARS associated with a decrease in plasma α-tocopherol levels (Wander et al., 1997). We did not see a decrease in α-tocopherol in our study which may explain this discrepancy.

The main conclusion from this study was that feeding a diet containing an (n-6) : (n-3) ratio of 5 : 1 did not have a negative effect on the immune response of young or geriatric dogs. If anything there was an increase in T- and B-cell activation in the cells isolated from the dogs fed an (n-6) : (n-3) ratio of 5 : 1. Several interesting breed and age differences were observed in this study. First, the metabolism of n-3 fatty acids appears to be differentially regulated by breed and age in the dog. These breed-associated differences in fatty acid metabolism may explain why not all atopic dogs respond to fatty acid therapy. This suggests that consideration of specific dietary fatty-acid profiles may be warranted for different breeds and stages of life. Secondly, T- and B-cell proliferative ability appears to be lower in Labrador Retrievers than in Fox Terriers. How this effects the health of these two breeds will require further study.

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